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Award Number: DAMD17-02-1-0566

TITLE: Studies on the Novel Anticancer Agents Metabolically Formed from 17-beta-Estradiol

PRINCIPAL INVESTIGATOR: Anthony J. Lee

CONTRACTING ORGANIZATION: University of South Carolina
Research Foundation
Columbia, SC 29208

REPORT DATE: June 2004

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 2004	Annual Summary (1 Jun 2002 - 31 May 2004)

4. TITLE AND SUBTITLE
Studies on the Novel Anticancer Agents Metabolically Formed from 17-beta-Estradiol

5. FUNDING NUMBERS
DAMD17-02-1-0566

6. AUTHOR(S)

Anthony J. Lee

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of South Carolina
Research Foundation
Columbia, SC 29208

E-Mail: anthonyl@mail.med.upenn.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

This is the **final report** for my Predoctoral Traineeship Award (No. DAMD17-02-1-0566). The studies described in the original grant proposal have been completed. Our major findings included the following: (1) We demonstrated, for the first time, that a novel class of nonpolar estradiol (E_2) metabolites were formed by human liver microsomes and also by certain human cytochrome P450 (CYP) enzymes using NADPH as a cofactor. (2) Among a total of some 20 nonpolar E_2 metabolite peaks detected, M15 and M16 were only selectively formed with a few of the human CYP isoforms (namely CYP3A4, CYP3A5, CYP1A1, CYP2C8, and CYP2C9). The formation of these two representative nonpolar estrogen metabolites by human CYP isoforms did not correlate with their overall catalytic activity for the oxidative metabolism of E_2. (3) The structures of the metabolically-formed M15 and M16 were unequivocally identified to be the dimers of E_2, linked together through a diaryl ether bond between a phenolic oxygen atom of one E_2 molecule and the 2- or 4-position aromatic carbon of another E_2. (4) M15 and M16 were chemically synthesized by using estradiol as the starting material.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
9

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Introduction

This is the **final report** for the Predoctoral Traineeship Award (No. DAMD17-02-1-0566). Recently, the PH.D. Dissertation Committee of our graduate program approved me for graduation with a PH.D. Degree in Basic Pharmaceutical Sciences. The studies described in the original grant proposal have been completed.

Body

The Specicific Aims of the Original Proposal:

1. *To determine the chemical structure of X2 (a major anticancer nonpolar E_2 metabolite) by using various chemical and analytical methods.*

2. *To evaluate each of the nonpolar E_2 metabolites formed by human liver microsomes for their inhibitory effects on the proliferation of ER(+) human breast cancer cell lines (MCF-7, T-47D, and ZR-75-1) and ER(-) human breast cancer cell lines (MDA-MB-231 and MDA-MB-435s).*

3. *To characterize the complete profiles of the nonpolar E_2 metabolites formed by representative human liver and placental microsomes.*

   Significance: Studies described in this application will characterize the metabolic profiles of various nonpolar E_2 metabolites that are formed by human tissues. More importantly, I will determine the biological activities of various nonpolar E_2 metabolites for inhibiting the growth of human breast cancer cell lines. The structural information of the endogenous anti-breast cancer compound (X2) will be useful for the chemical synthesis of large amounts of this compound for further testing of its actions in animal models and eventually in humans for the treatment of breast cancers. I believe that success of my proposed studies will form the basis for future research efforts to further understand the mechanisms of their actions and to develop these novel estrogen metabolites as potential anti-breast cancer agents.

Major findings:

1. By using a versatile HPLC method (total elution time ~135 min) I developed, I detected the formation of some 20 nonpolar radioactive metabolite peaks (designated as M1 through M20), in addition to a large number of polar hydroxylated or keto metabolites, following incubations of [^{3}H]17β-estradiol with human liver microsomes or cytochrome P450 3A4 in the presence of NADPH as a cofactor. The formation of most of the nonpolar estrogen metabolite peaks (except M9) was dependent on the presence of human liver microsomal proteins, and could be selectively inhibited by the presence of carbon monoxide. Among the four cofactors (NAD, NADH, NADP, NADPH) tested, NADPH was the optimum cofactor for the metabolic formation of polar and nonpolar estrogen metabolites in vitro, although NADH also had a weak ability to support the

reactions. These observations suggest that the formation of most of the nonpolar estrogen metabolite peaks requires the presence of liver microsomal enzymes and NADPH. Chromatographic analyses showed that these nonpolar estrogen metabolites were not the monomethyl ethers of catechol estrogens or the fatty acid esters of 17β-estradiol. Analyses using liquid chromatography/mass spectrometry (LC/MS) and nuclear magnetic resonance (NMR) showed that M15 and M16, two representative major nonpolar estrogen metabolites, are diaryl ether dimers of 17β-estradiol. These data suggest a new metabolic pathway for the NADPH-dependent, microsomal enzyme-mediated formation of estrogen diaryl ether dimers, along with other nonpolar estrogen metabolites.

2. I also characterized the NADPH-dependent formation of some 20 nonpolar estrogen metabolites by fifteen human CYP isoforms (CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 3A4, 3A5, 3A7, and 4A11). I found that some of the nonpolar metabolites were formed at varying rates with each of the 20 human CYP isoforms tested, but M15 and M16 were selectively formed only with a few CYP isoforms. CYP3A4 and 3A5 had the highest catalytic activity for the formation of M15 and M16, but CYP1A1, 2C8 and 2C9 had weak but detectable catalytic activity for their formation. Kinetic analyses showed that the apparent K_M values for CYP3A4 and CYP3A5-dependent formation of M15 and M16 ranged from 46–119 μM, and their apparent V_{MAX} values ranged from 206–276 pmol/nmol of CYP/min. Using mass and NMR spectrometric analyses, we unequivocally identified the structures of M15 and M16 to be the dimers of 17β-estradiol, which were connected together through a diaryl ether bond between a phenolic oxygen atom of one 17β-estradiol molecule and the 2- or 4-position aromatic carbon of the other 17β-estradiol.

3. Using estradiol as the starting material, I designed a four-step method for the chemical synthesis of these two estrogen dimmers with the Ullmann condensation reaction as a key step: STEP 1: Synthesis of 2- or 4-bromoestradiol from estradiol. STEP 2: Protection of the C-3 phenolic hydroxyl group of the 2- or 4-bromoestradiol. STEP 3: The Ullmann condensation reaction between the phenol-protected bromoestradiol and the estradiol potassium salt under our modified reaction conditions (with a 41% product yield). STEP 4: Removal of the C-3 benzyl group by catalytic hydrogenation. The chromatographic and various spectrometric properties of the two synthesized compounds were identical to those metabolically formed by human cytochrome P450 3A4.

Key Research Accomplishments

1. We have completed studies aimed at determining the chemical structures of M15 and M16 (originally called X1 and X2 in the grant proposal) by using various chemical and analytical methods.

2. We have chemically synthesized M15 and M16.

3. We have completed testing the inhibitory effects of M15 and M16 on the proliferation of ER(+) human breast cancer cell lines (MCF-7, T-47D, and ZR-75-1) and ER(-) human breast cancer cell lines (MDA-MB-231 and MDA-MB-435s).

4. We have systematically characterized the nonpolar E_2 metabolites formed by human liver microsomes and formed by 15 human cytochrome P450 isoforms.

Reportable Outcomes

Listed below are papers and abstracts that have come out of this award, with the P.I.'s name highlighted.

Lee AJ, Kosh JW, Conney AH and Zhu BT [2001] Characterization of the NADPH-dependent metabolism of 17β-estradiol to multiple metabolites by human liver microsomes and selectively-expressed human cytochrome P450 3A4 and 3A5. Journal of Pharmacology and Experimental Therapeutics 298: 420-432.

Mills LH, **Lee AJ** and Parlow AF and Zhu BT [2001] Preferential growth stimulation of mammary glands over uterine endometrium in female rats by a naturally occurring estradiol-17β-fatty acid ester. Cancer Research 61: 5764-5770.

Zhu BT, Patel UK, Cai MX, **Lee AJ** and Conney AH [2001] Rapid conversion of tea catechins to monomethylated products by rat liver cytosolic catechol-O-methyltransferase. Xenobiotica 31: 879-890.

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Lee AJ, Cai MX, Thomas PE, Conney AH and Zhu BT [2003] Characterization of the oxidative metabolites of 17β-estradiol and estrone formed by fifteen selectively-expressed human cytochrome P450 isoforms. Endocrinology 144: 3382-3398.

Lee AJ, Conney AH and Zhu BT [2003] Human cytochrome P450 3A7 has a distinct high catalytic activity for the 16α-hydroxylation of estrone, but not 17β-estradiol. Cancer Research 63: 6532-6536.

Lee AJ, Pellechia PJ, Walla MD and Zhu BT [2004] Characterization of a novel class of nonpolar 17β-estradiol metabolites formed by human cytochrome P450 enzymes. Medical Hypotheses and Research 1: 53-65.

Lee AJ, Sowell JW, Cotham WE and Zhu BT [2004] Chemical synthesis of two novel diaryl ether dimers of estradiol-17β. Steroids 69: 61-65.

Hook LL, **Lee AJ** and Zhu BT [2000] Differential stimulatory actions of estradiol-17β-stearate on the growth of rat mammary vs. uterine cells. Proceedings of the American Association for Cancer Research 41: 429, San Francisco.

Lee AJ, Conney AH and Zhu BT [2000] NADPH-dependent metabolism of 17β-estradiol and estrone by twenty-four human liver microsomes. Proceedings of the American Association for Cancer Research 41: 743, San Francisco, California.

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Lee AJ, Liu ZJ and Zhu BT [2002] Lack of biological activity of 2-methoxyestradiol-3-sulfate and its differential metabolic formation in human breast cancer cell lines. Proceedings of the American Association for Cancer Research 43: 424, San Francisco, California.

Liu ZJ, **Lee AJ** and Zhu BT [2002] Resistance of ZR-75-1 human breast cancer cells to the anticancer actions of 2-methoxyestradiol: 17β-HSD as a possible mechanism. Proceedings of the American Association for Cancer Research 43: 1090, San Francisco, California.

Mills LH, **Lee AJ** and Zhu BT [2003] Naturally-occurring estradiol-17β-fatty acid ester, but not estradiol-17β, preferentially induces the development of mammary tumor in female ACI rats. Proceedings of the American Association for Cancer Research 44: 835-836.

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Conclusions

1. We demonstrated, for the first time, that a novel class of nonpolar E_2 metabolites were formed by human liver microsomes and also by certain human CYP enzymes using NADPH as a cofactor.

2. Among a total of some 20 nonpolar E_2 metabolite peaks detected, M15 and M16 were only selectively formed with a few of the human CYP isoforms (namely CYP3A4, CYP3A5, CYP1A1, CYP2C8, and CYP2C9). The formation of these two representative nonpolar estrogen metabolites by human CYP isoforms did not correlate with their overall catalytic activity for the oxidative metabolism of E_2.

3. The structures of the metabolically-formed M15 and M16 were unequivocally identified to be the dimers of E_2, linked together through a diaryl ether bond between a phenolic oxygen atom of one E_2 molecule and the 2- or 4-position aromatic carbon of another E_2.

4. M15 and M16 were chemically synthesized by using estradiol as the starting material.

References

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Appendices

Not included.